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ORIGINAL RESEARCH

Clinical Outcomes of Autologous Stem Cell–Patch Implantation for Patients With Heart Failure With Nonischemic Dilated Cardiomyopathy

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BACKGROUND: Clinical effectiveness of autologous skeletal cell-patch implantation for nonischemic dilated cardiomyopathy has not been clearly elucidated in clinical settings. This clinical study aimed to determine the feasibility, safety, therapeutic efficacy, and the predictor of responders of this treatment in patients with nonischemic dilated cardiomyopathy.

METHODS AND RESULTS: Twenty-four nonischemic dilated cardiomyopathy patients with left ventricular ejection fraction <35% on optimal medical therapy were enrolled. Autologous cell patches were implanted over the surface of the left ventricle through left minithoracotomy without procedure-related complications and lethal arrhythmia. We identified 13 responders and 11 nonresponders using the combined indicator of a major cardiac adverse event and incidence of heart failure event. In the responders, symptoms, exercise capacity, and cardiac performance were improved postoperatively (New York Heart Association class II 7 [54%] and III 6 [46%] to New York Heart Association class II 12 [92%] and I 1 [8%], P<0.05, 6-minute walk test; 471 m [370–541 m] to 525 m [425–555 m], P<0.05, left ventricular stroke work index; 31.1 g·m^2·beat [22.7–35.5 g·m^2·beat] to 32.8 g·m^2·beat [28–38.5 g·m^2·beat], P=0.21). However, such improvement was not observed in the nonresponders. In responders, the actuarial survival rate was 90.9±8.7% at 5 years, which was superior to the estimated survival rate of 70.9±5.4% using the Seattle Heart Failure Model. However, they were similar in nonresponders (47.7±21.6% and 56.3±8.1%, respectively). Multivariate regression model with B-type natriuretic peptide, pulmonary capillary wedge pressure, and expression of histone H3K4me3 (H3 lysine 4 trimethylation) strongly predicted the responder of this treatment (B-type natriuretic peptide: odds ratio [OR], 0.96; pulmonary capillary wedge pressure: OR, 0.58; H3K4me3: OR, 1.35, receiver operating characteristic–area under the curve, 0.96, P<0.001).

CONCLUSIONS: This clinical trial demonstrated that autologous skeletal stem cell–patch implantation might promise functional recovery and good clinical outcome in selected patients with nonischemic dilated cardiomyopathy, in addition to safety and feasibility.

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Key Words: cardiac regenerative therapy ■ heart failure ■ nonischemic dilated cardiomyopathy

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The number of patients with heart failure is increasing throughout the world. Pharmacologic therapy along with pacemakers and implantable cardiac defibrillators have important roles in treatment for affected patients. In addition, heart transplantation and use of a left ventricular assist device (LVAD) as a bridge

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CLINICAL PERSPECTIVE

What Is New?

- This is the first clinical trial of autologous skeletal stem cell–patch implantation evaluating its feasibility, safety, and efficacy for the patients with nonischemic dilated cardiomyopathy.
- This study also revealed that the combination of B-type natriuretic peptide, pulmonary capillary wedge pressure, and H3K4me3 (H3 lysine 4 trimethylation) strongly predicted the responders of this treatment.

What Are the Clinical Implications?

- The responders showed improvement of symptoms, exercise capacity, and cardiac performance after cell-patch implantation.
- The responders also showed better actuarial survival rate compared with estimated survival rate using the Seattle Heart Failure Model.
- The autologous stem cell–patch implantation might promise functional recovery and good clinical outcome in selected patients with nonischemic dilated cardiomyopathy.

Nonstandard Abbreviations and Acronyms

6MWD	6-minute walk distance test
DCM	dilated cardiomyopathy
dHCM	dilated phase of hypertrophic cardiomyopathy
H3K4me2	human histone H3 lysine 4 demethylation
H3K4me3	human histone H3 lysine 4 trimethylation
H3K9ac	human histone H3 lysine 9 acetylation
H3K9me2	human histone H3 lysine 9 demethylation
H3K9me3	human histone H3 lysine 9 trimethylation
hiPS-CMs	human induced pluripotent stem cell–derived cardiomyocytes
LVEDD	left ventricular end-diastolic dimension
LVSWI	left ventricular stroke work index
MACE	major adverse cardiac event
NIDCM	nonischemic dilated cardiomyopathy
NYHA-FC	New York Heart Association functional classification
PAP	pulmonary artery pressure
SAS	specific activity scale
SHFM	Seattle Heart Failure Model

to heart transplantation and destination therapy are indicated for progressive and advanced heart failure refractory to medical therapy, though they are limited by availability and durability worldwide. As for LVAD treatment, medical cost and complications related to the device remain a challenge, whereas heart transplantation is limited because of a serious shortage of donor hearts despite being shown to be the definitive treatment for heart failure.[1,2] Thus, widely applicable treatments are needed as alternatives or to complement the standard available treatments for advanced heart failure.

According to previously reported results from a phase I clinical trial that utilized autologous skeletal stem cell–patch implantation, possible efficacy of that treatment based on angiogenesis induced by secreted cytokines has been shown for patients with ischemic cardiomyopathy, resulting in approval by the health ministry in Japan for clinical use.[3,4] More recently, studies have demonstrated that the mechanism of efficacy of autologous skeletal stem cells for treating a failing heart is not only because of angiogenesis, but also antifibrotic action or preservation of cytoskeletal proteins in myocytes, which were shown to suppress functional deterioration in delta-sarcoglycan– deficient dilated cardiomyopathy (DCM) hamsters and a rapid pacing DCM canine model.[5,6] Evidence of related mechanisms noted in other reports has led to a proposal that use of a skeletal stem cell patch in clinical situations may provide healing or at least benefits for human nonischemic DCM (NIDCM).[3] On the other hand, NIDCM has a diverse pathogenesis and distinct subtypes sharing a common phenotype; thus, some individuals with NIDCM may show a better response to this regenerative therapy, and it is crucial to determine which show responsiveness.[7]

In the present study, we examined the feasibility, safety, and potential therapeutic efficacy of autologous skeletal cell-patch implantation, and aimed to reveal factors that identify potential responders to this treatment among patients with NIDCM.

METHODS

This study was approved by the institutional review committee of Osaka University Graduate School of Medicine, Osaka, Japan. Informed consent was obtained from each patient before their participation in the study. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Clinical Trial Registration Information

This study was conducted according to the Guidelines on Clinical Research Using Human Stem Cells from

the Japanese Ministry of Health, Labor and Welfare (UMIN ID: UMIN000012906; https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000015089, UMIN000015892; https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000016519, UMIN00003273; https://upload.umin.ac.jp/cgi-open-bin/ctr/ctr_view.cgi?recptno=R000003959).

Patient Characteristics

After giving written informed consent for participation, 24 patients were enrolled from April 2010 to March 2017. Prior to registration, all underwent cardiac echocardiography, coronary angiography, and cardiac biopsy examinations to confirm the cause of the cardiomyopathy. According to the examinations, the cause of NIDCM was idiopathic dilated cardiomyopathy in 21 patients, dilated phase of hypertrophic cardiomyopathy (dHCM) in 2 patients, and postmyocarditis in 1 patient. Patients with NIDCM were considered eligible according to the following criteria: 20 to 75 years of age, ejection fraction under 35% as evaluated by cardiac echocardiography, New York Heart Association functional classification (NYHA-FC) of 2 or more, and already received optimal medical treatment including surgical and/or cardiac resynchronization–defibrillator therapy with a defibrillator. Patients with malignancy, end-stage renal failure requiring hemodialysis, infectious disease, or LVAD support were excluded.

As for the NYHA-FC, 8 patients (33.3%) were NYHA-FC 2, and 15 patients (62.5%) were NYHA-FC 3. One patient was receiving continuous catecholamine injections and was considered to be NYHA-FC 4. Eleven of the patients had received cardiac resynchronization–defibrillator therapy, and 5 an implantable cardiac defibrillator. Ten patients underwent mitral valve surgery at >4 months before cell-patch implantation (Table 1). We did not change medications or reset the condition of the resynchronization device after performing autologous stem cell–patch implantation in any of the enrolled patients. Average follow-up was 47.5±4.3 months.

Table 1. Patients' Characteristics

Demographics	All Cohort, n=24	Responders, n=13	Nonresponders, n=11	*P* Value
Age, y	53.5±2.6	53.5±4.3	53.5±3.0	0.68
>65 y	5 (20.8%)	4 (30.8%)	1 (9.1%)	0.19
Men	22 (91.7%)	12 (92.3%)	10 (90.9%)	0.90
Risk factor				
Hypertension	4 (16.7%)	3 (23.1%)	1 (9.1%)	0.36
Hyperlipidemia	3 (12.5%)	1 (7.7%)	2 (18.2%)	0.44
Diabetes mellitus	1 (4.2%)	0 (0%)	1 (9.1%)	0.27
Oral medication	1 (4.2%)	0 (0%)	1 (9.1%)	0.27
Cardiac history				
ICD implantation	5 (20.8%)	2 (15.4%)	3 (27.3%)	0.47
CRT-D implantation	11 (45.8%)	5 (38.5%)	6 (54.6%)	0.43
History of surgical intervention				
Mitral valve surgery	10 (41.7%)	5 (38.5%)	5 (45.5%)	0.73
CABG	1 (4.2%)	0 (0%)	1 (9.1%)	0.27
Aortic valve surgery	1 (4.2%)	0 (0%)	1 (9.1%)	0.27
IABP	0 (0.0%)	0 (0%)	0 (0%)	1.00
LVAD	0 (0.0%)	0 (0%)	0 (0%)	1.00
Medication				
ACE-I	15 (62.5%)	7 (53.9%)	8 (72.7%)	0.34
ARB	2 (8.3%)	2 (15.4%)	0 (0%)	0.17
β-blocker	24 (100%)	13 (100%)	11 (100%)	1.00
Diuretics	22 (91.7%)	12 (92.3%)	10 (90.9%)	0.90
Antiplatelet	5 (20.8%)	3 (23.1%)	2 (18.2%)	0.77
Warfarin	18 (75.0%)	11 (84.6%)	7 (63.6%)	0.24
Amiodarone	10 (41.7%)	8 (61.5%)	2 (18.2%)	0.03
Statins	3 (12.5%)	1 (7.7%)	2 (18.2%)	0.44
Continuous catecholamine infusion	1 (4.2%)	0 (0%)	1 (9.1%)	0.27

ACE-I indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CABG, coronary artery bypass grafting; CRT-D, cardiac resynchronization therapy with defibrillator; IABP, intra-aortic balloon pump; ICD, implantable cardioverter defibrillator; and LVAD, left ventricular assist device.

Culture and Fabrication of Cell Patches From Autologous Skeletal Muscle

Muscle specimens were harvested from vastus medialis muscle tissue. Muscle fibers were collected by removing connective tissues with collagenase and TrypLE Select (Invitrogen), then suspended in MCDB131 medium (Invitrogen) with 20% fetal bovine serum. Cell suspensions were cultured for 4 weeks, and total myoblast cell numbers were calculated as follows: CD56 positive rate×viability rate×total cell number. The criteria for determining the quality of myoblast cells were as follows: negative for sterilization, endotoxin and mycoplasma tests, and number not lower than 1.8×10^8. When cell quality was not up to those standards, we harvested additional muscle tissue from the opposite side of the vastus medialis muscle and used the same culture process. Cell suspensions were placed in temperature-responsive cell-culture dishes (UpCell, Cell Seed, Japan), the surface of which contained a temperature-responsive polymer (poly-N-isopropylacrylamide).[8] After the temperature was reduced to 32°C, the surface rapidly became hydrated, prompting complete detachment of adherent cells as a cell patch. After detachment from the temperature-responsive dishes, the top surface of each cell patch was reinforced by fibrin glue. Each cell patch was ≈50 mm in diameter and contained 6.0×10^7 cells.

Cell-Patch Implantation

Under general anesthesia and single-sided ventilation, the fifth or sixth intercostal space was opened, and the pericardium was opened parallel to the phrenic nerve. Because we approached through a left thoracotomy, the posterior wall of the left ventricle (LV) was difficult to expose; therefore, the anterior and lateral walls of the LV were dissected as widely as possible. Then 5 to 6 cell patches were placed and fixed with 7-0 Prolene suture so that the whole anterior and lateral LV walls were covered by the cell patches.

Histological Analysis

Sampled cardiac tissue was fixed with 10% buffered formalin and embedded in paraffin. Transverse sections of LV tissue (2-μm thick) were subjected to periodic acid-Schiff and Masson trichrome staining. The transverse diameter of cardiac myocytes in periodic acid-Schiff–stained sections was measured, and the percentage of fibrotic area in the cardiac tissue in Sirius red–stained sections was determined using a planimetric method with Windows MetaMorph software (Universal Imaging, Downingtown, PA). For immunohistochemical labeling, paraffin-embedded sections were deparaffinized in xylene, dehydrated in graded ethanol mixtures, and processed for antigen retrieval by autoclaving in 0.01 mol/L citrate buffer.

The sections were then immersed in methanol containing 3% hydrogen peroxide and incubated with mouse monoclonal primary antibodies against human histone H3K4 (H3 lysine 4) H3K4me2 (demethylation), H3K4me3 (H3K4 trimethylation), histone H3K9 (H3 lysine9) H3K9me2 (demethylation), H3K9me3 (H3K9 trimethylation), and H3K9ac (H3K9 acetylation). The primary antibodies were purchased from Abcam (Cambridge, UK). Subsequently, the sections were incubated with a biotinylated anti-mouse immunoglobin G antibody (Dako, Glostrup, Denmark) and further with peroxidase-conjugated streptavidin (Dako), then visualized with the use of 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (Wako Pure Chemical Industries, Tokyo, Japan).[9–11] Six different fields per sample were randomly selected, and 50 to 100 myocytes per field were examined under a light microscope (Leica, Wetzlar, Germany). Consequently, 300 to 600 myocytes were measured per sample. Six different fields were randomly selected by 2 of the authors (E.I., H.I.), and the number of antibody-positive cells in each field was counted by a different pair of authors (M.T., A.H.), who were blinded to the sources of the samples.

Study End Points

Feasibility and Safety

The feasibility of the present treatment was determined on the basis of the procedural success of culture, fabrication, and implantation of the cell patch. Safety of the treatment was examined by noting the occurrence of a procedure-related complication or a major adverse cardiac event (MACE) up to 6 months after implantation. A MACE was defined as a composite of death caused by cardiac failure, sudden death from unknown reason, deterioration of cardiac function requiring LVAD support or continuous catecholamine support, myocardial infarction, and lethal arrhythmias. To monitor arrhythmia, all patients underwent 24-hour monitoring during hospitalization, as well as Holter ECG examinations performed at 6 months and 1 year after cell-patch implantation.

Blood sampling test including B-type natriuretic peptides (BNP) was also performed before, 1 week, 1, 3, and 6 months, and 1 year after treatment to evaluate adverse events caused by cell-patch implantation.

Evaluation of Heart Failure Event

A heart failure event was defined as admission according to the Framingham heart failure diagnostic criteria.[12] A preoperative heart failure event was retrospectively

evaluated on the basis of medical records, and duration of heart failure was defined as time from the first preoperative heart failure event to cell-patch implantation. The incidence of heart failure was determined on the basis of the number of heart failure events and duration of heart failure.

Cardiac Echocardiography

An echocardiographic study was performed for all patients before, 6 months, and 1 year after treatment. Left ventricular ejection fraction (LVEF), left ventricular end-diastolic dimension (LVEDD), and left ventricular end-systolic dimension were evaluated, with LVEF calculated using the modified Simpson method.

Right Heart Catheterization

Right heart catheterization was performed with an internal vein approach using a Swan-Ganz catheter to obtain right atrial pressure, pulmonary artery pressure (PAP), and pulmonary capillary wedge pressure (PCWP). We calculated cardiac output based on thermodilution, whereas pulmonary vascular resistance (PVR) was calculated as follows: PVR=(mean PAP–mean PCWP)/cardiac output×79.92 (dyne·s·cm^{-5}). Furthermore, left ventricular stroke work index (LVSWI) was calculated as follows: LVSWI=cardiac output/heart rate×(mean arterial pressure–PCWP)×13.6/body surface area (g·m^2·beat).

Evaluation of Symptoms and Exercise Capacity

We evaluated the exercise capacity in each patient using a 6-minute walk distance (6MWD) test and the Japanese version of the specific activity scale (SAS) before, 6 months, and 1 year after cell-patch implantation.[13] The 6MWD test measures the distance a patient is able to walk over a total of 6 minutes on a flat floor, and SAS is determined by metabolic equivalents calculated based on an interview conducted by a physician.[14,15] We also evaluated the correlation between the subjectively assessed SAS and objectively measured 6MWD test as the indicators of exercise capacity. Changes in symptoms were evaluated using the NYHA-FC before, 6 months, and 1 year after cell-patch implantation.

Evaluation of Efficacy of Skeletal Myoblast Cell-Patch Implantation

The efficacy of the cell-patch treatment was determined on the basis of a MACE and incidence of heart failure. Therefore, we defined responders to this treatment as patients who did not develop a MACE during the follow-up period or whose incidence of heart failure events did not increase after the treatment as compared with the preoperative value. Serial changes of all variables were evaluated in the responder and nonresponder groups. The freedom from all-cause mortality rate was also evaluated and compared with predicted survival rate calculated by the Seattle Heart Failure Model (SHFM), which was the average of the predicted survival of each case at 1, 3, and 5 years.

Statistical Analysis

All continuous variables were expressed as the mean±standard error or median (interquartile range), as indicated. Serial changes of all variables including incidence of heart failure event between the pre- and posttreatment period were evaluated using a Wilcoxon signed rank test.

Even if the test was repeated at multiple time points, it was decided not to correct multiple comparison because of the exploratory analyses. For repeatedly measured data, missing values were imputed from the last observation carried forward without other imputation procedures. Complete-case analyses were also performed as a sensitivity analysis for repeatedly measured data. Correlation between the SAS and 6MWD test was evaluated using the Spearman rank correlation coefficient. Histological variables were compared with the use of the Mann-Whitney U test for the 2 groups. Actuarial estimates of all-cause mortality and MACE were calculated using the Kaplan-Meier method. Logistic regression analysis was performed to identify the variables related to the responder. The receiver operating characteristic curve was calculated for evaluating the predictive accuracy of factors of the responder after cell-patch implantation. A P value of <0.05 was considered to indicate significance. Statistical analyses were performed using JMP 8.0 software (SAS Institute, Cary, NC). These statistical analyses were performed by a third party for unmasking.

RESULTS

Feasibility and Safety Analysis

Muscle specimens were harvested from each patient without complications (Figure 1A). In 20 patients, the quality of the initially obtained myoblast cells matched the criteria described above, whereas 4 (16.7%) required second muscle harvesting and culturing because of a low number of myoblast cells. Nevertheless, all patients successfully underwent cell-patch implantation without any procedure-related complications (Figure 1B and 1C). The average number of transplanted myoblast cells was 3.9±0.4×10^8. Postoperatively, all patients required temporary intravenous catecholamine support, and 2 patients required prolonged catecholamine support because of severely deteriorated preoperative cardiac function. However, all patients were

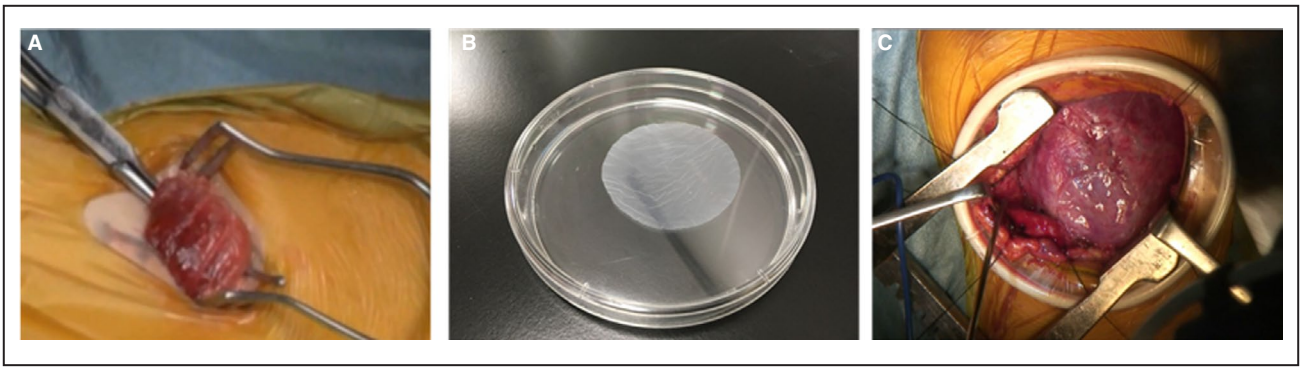

Figure 1. Fabrication and implantation of the cell patch.
A, Muscle specimens were harvested from vastus medialis muscle. **B**, The appearance of cell patches. **C**, Cell patches were implanted to the left ventricular free wall via left minithoracotomy.

discharged to home at an average 47.3±10.6 days following the procedure. During hospitalization, lethal arrhythmias, such as sustained ventricular tachycardia and ventricular fibrillation, were not observed in any of the patients. Two patients had a heart failure event within 6 months after treatment, whereas no other MACE occurred within 6 months after treatment. No sustained ventricular tachycardia and ventricular fibrillation was observed by the Holter ECG examination at 6 months and 1 year after cell-patch implantation and implantable cardioverter defibrillator records.

Efficacy of Skeletal Myoblast Cell-Patch Implantation Evaluated by a MACE and Incidence of Heart Failure

During the follow-up period, 10 MACE incidents were noted, in which 8 patients underwent LVAD implantation and 1 depended on intravenous catecholamine support. One patient died of arrhythmia at 31 months after undergoing cell-patch implantation. Because this event occurred 31 months after treatment, we judged that it was not related to the procedure but rather the progressive course of the disease.

Before the operation, there were 48 heart failure events, for a preoperative incidence of heart failure of 0.70±0.14 events per year on average. Following cell-patch implantation, we encountered 20 heart failure events, for a postoperative incidence of heart failure of 0.69±0.20 events per year. After the procedure, the incidence of heart failure increased in 7 patients (29.2%).

Following implantation, a MACE was found in 10 patients, and an increase in incidence of heart failure was found in 7, with 6 patients experiencing both during the follow-up period. Thus, 13 (54.2%) patients were considered to be responders and 11 (45.8%) were considered to be nonresponders to this treatment (Tables 2

Table 2. Clinical Result of Responders

Case	Age, y	Pathogenesis	NYHA-FC	LVEF	MACE	Increasing the incidence of HF	Result
1	70	Idiopathic	3	20	No	-	Deceased
2	67	Idiopathic	3	25	No	-	Deceased
3	67	Idiopathic	3	23	No	-	Alive
4	66	Idiopathic	2	33	No	-	Alive
5	63	Idiopathic	2	35	No	-	Alive
6	63	Idiopathic	2	18	No	-	Alive
7	59	Idiopathic	3	15	No	-	Alive
8	50	Idiopathic	3	19	No	-	Alive
9	50	Idiopathic	2	21	No	-	Alive
10	46	Idiopathic	2	31	No	-	Alive
11	45	Idiopathic	3	21	No	-	Alive
12	28	Idiopathic	2	19	No	-	Alive
13	21	Idiopathic	2	20	No	-	Alive

HF indicates heart failure; LVEF, left ventricular ejection fraction; MACE, major adverse cardiac event; and NYHA-FC, New York Heart Association functional classification.

Table 3. Clinical Result of Nonresponders

Case	Age, y	Pathogenesis	NYHA-FC	LVEF	MACE	Duration From Implantation, mo	Increasing the Incidence of HF	Result
1	68	Idiopathic	3	7	CA	6	+	Deceased
2	62	Idiopathic	3	10	LVAD	10	–	Alive
3	61	Idiopathic	3	18	LVAD	30	–	Alive
4	54	Idiopathic	3	20	LVAD	13	+	Alive
5	53	Idiopathic	3	14	LVAD	6	+	Alive
6	46	Idiopathic	3	20	LVAD	14	+	Alive
7	36	Idiopathic	4	22	Arrhythmia	31	–	Deceased
8	60	dHCM	2	34	LVAD	11	+	Deceased
9	58	dHCM	3	22	LVAD	31	–	Deceased
10	39	Postmyocarditis	3	23	LVAD	16	+	Alive
11	51	Idiopathic	3	9	No	–	+	Alive

CA indicates intravenous catecholamine support; dHCM, dilated phase of hypertrophic cardiomyopathy; HF, heart failure; LVAD, left ventricular assist device; LVEF, left ventricular ejection fraction; MACE, major adverse cardiac event; and NYHA-FC, New York Heart Association functional classification.

and 3). In the responder group, none developed a heart failure event after cell-patch implantation, and the incidence of heart failure significantly decreased from 0.74±0.21 to 0 events per year (P<0.01). On the other hand, the incidence of heart failure in the nonresponder group significantly increased from 0.64±0.18 to 1.51±0.29 events per year after treatment (P<0.05).

Rates of Freedom From All-Cause Mortality, MACE, and Heart Failure

There were 6 late deaths attributable to a cerebrovascular accident in 4 patients, in whom 2 cerebrovascular accidents were attributable to LVAD complication, arrhythmia in 1, and heart failure in 1. For all patients, the freedom from all-cause mortality rate was 95.8±4.1% at 1 year, 86.8±7.1% at 3 years, and 68.4±14.2% at 5 years. Average predicted survival rate using the SHFM in all cohorts was 91.0±1.9%, 76.3±3.8%, and 64.2±4.9% at 1, 3, and 5 years, respectively (Figure 2A). In the responder group, the freedom from all-cause mortality rate was 100% at both 1 and 3 years and 90.9±8.7% at 5 years, and it was superior to the estimated survival rate using the SHFM at each point (93.8±1.4%, 81.6±3.6%, and 70.9±5.4% at 1, 3, and 5 years, respectively). On the other hand, the freedom from all-cause mortality rate was 90.9±8.7%, 71.6±14.0%, and 47.7±21.6% at 1, 3, and 5 years, respectively, in the nonresponder group, which was similar to the estimated survival rate using the SHFM (87.6±3.6%, 70.0±6.8%, and 56.3±8.1% at 1, 3, and 5 years, respectively) (Figure 2A and 2B). In the responder group, there was no incidence of a MACE or heart failure, which resulted in 100% freedom from a MACE and heart failure events at 1, 3, and 5 years. In the nonresponder group, the rates of freedom from a MACE were 63.4% and 9.1% at 1 and 3 years after the procedure, respectively. All MACE events occurred within 3 years after the procedure in the nonresponder group.

Functional Changes After Cell-Patch Implantation

In the responder group, neither LVEDD nor global LVEF deteriorated at 1 year after treatment (LVEDD: 71 mm [66–78 mm] to 68 mm [66–79 mm], P=0.64; LVEF: 21% [19%–25%] to 24% [195%–25%], P=0.92), and 6 of those 13 patients (46.2%) showed an increase in global LVEF. In the nonresponder group, LVEDD was not improved from 80 mm (66.5–81.5 mm) to 79 mm (69–82 mm) at 1 year after treatment (P=0.27). Furthermore, global LVEF in the nonresponder group significantly decreased from 20% (12%–22%) to 13% (8.5%–19.5%) at 6 months and 14% (11.5%–16.5%) at 1 year after treatment (P<0.05, P<0.05, respectively) (Figure 3A and 3B).

Right Heart Catheterization

In the nonresponder group, mean PAP, PCWP, and PVR values were significantly increased at 1 year after treatment, as follows: mean PAP, 21 mm Hg (16–26 mm Hg) to 25 mm Hg (20–39 mm Hg) (P<0.05); PCWP, 15 mm Hg (11–20.5 mm Hg) to 21 mm Hg (13–25 mm Hg) (P<0.05); and PVR, 142 dyne·s·cm^{-5} (92–179 dyne·s·cm^{-5}) to 187 dyne·s·cm^{-5} (124–286 dyne·s·cm^{-5}) (P<0.05). On the other hand, the mean PAP and PCWP values in the responder group did not significantly increase, and PVR decreased at 1 year after treatment, as follows: mean PAP 15 mm Hg (13–16 mm Hg) to 15 mm Hg (14–17 mm Hg) (P=0.31); PCWP, 8 mm Hg (6–10 mm Hg) to 8 mm Hg (8–10 mm Hg) (P=0.78); and PVR, 152 dyne·s·cm^{-5} (112–156 dyne·s·cm^{-5}) to 113 dyne·s·cm^{-5} (108–132 dyne·s·cm^{-5}) (P=0.22) (Figure 4A through 4C). Furthermore, LVSWI and cardiac index, good indicators of cardiac performance, increased at 1 year after

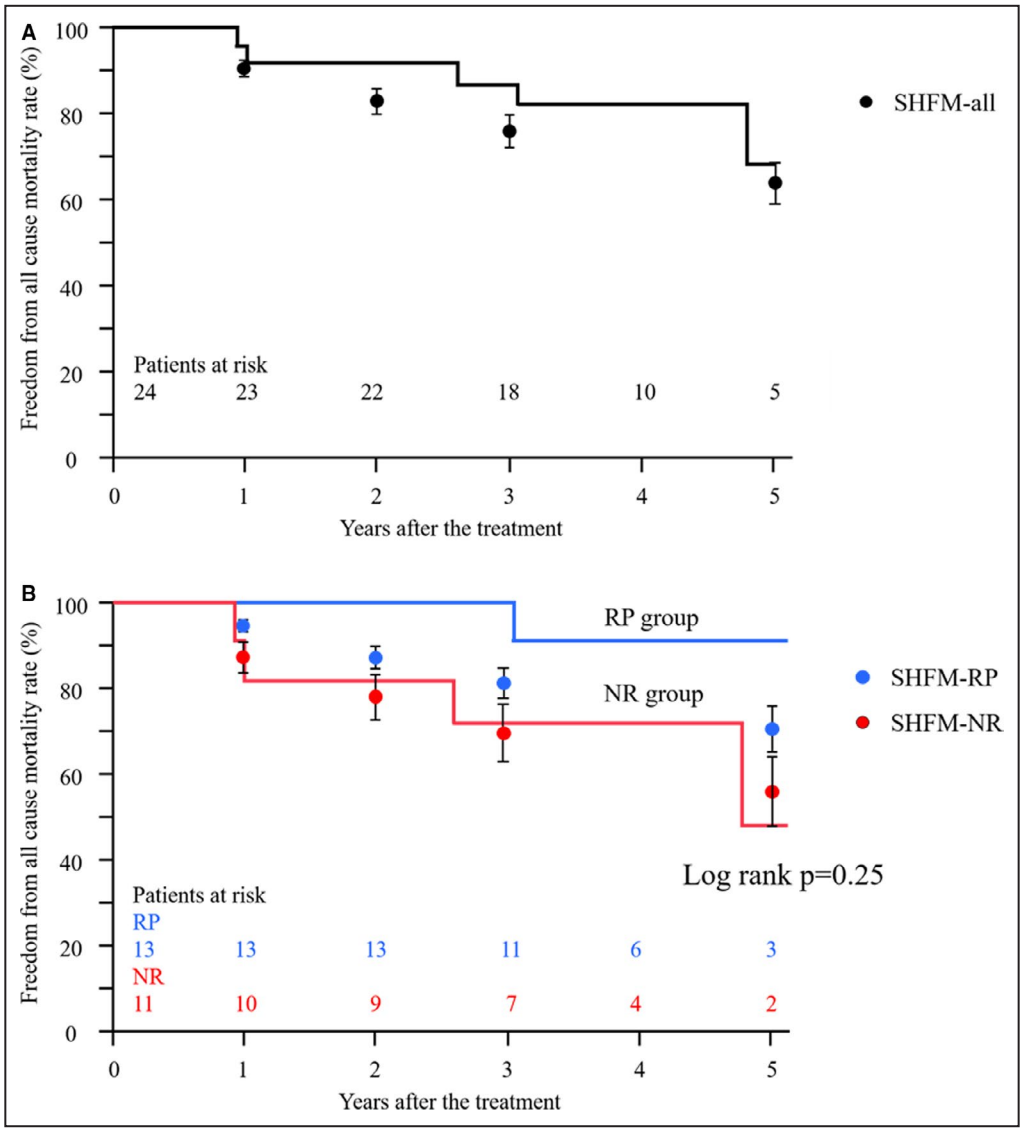

Figure 2. Freedom from all-cause mortality and predicted survival rate calculated by the Seattle Heart Failure Model (SHFM).
A, Freedom from all-cause mortality and predicted survival rate calculated by the SHFM in all cohorts. Kaplan-Meier estimate was plotted from observed data (solid black line). Average predicted survival rate was plotted (black dot) with standard error (SE) at 1, 2, 3, and 5 years. **B**, Freedom from all-cause mortality and predicted survival rate calculated by the SHFM in the responder (RP) and nonresponder (NR) groups. Kaplan-Meier estimate of RP and NR is plotted from observed data (blue and red solid line, respectively). The average predicted survival rate of the RPs and NRs is plotted (blue and red dot, respectively) with SE at 1, 2, 3, and 5 years.

cell-patch implantation in the responder group (cardiac index, 2.1 L/min·m^2 [1.8–2.3 L/min·m^2] to 2.3 L/min·m^2 [2.0–2.4 L/min·m^2] [P=0.11], LVSWI, 31.1 g·m^2·beat [22.7–35.5 g·m^2·beat] to 32.8 g·m^2·beat [28–38.5 g·m^2·beat] [P=0.21]). However, in the nonresponder group, those parameters were significantly decreased at 1 year after the treatment (cardiac index, 1.8 L/min·m^2 [1.7–2.4 L/min·m^2] to 1.6 L/min·m^2 [1.4–2.0 L/min·m^2] [P<0.05]; LVSWI, 19.1 g·m^2·beat [16.5–25.9 g·m^2·beat] to 13.1 g·m^2·beat [10.8–20.4 g·m^2·beat] [P<0.05]) (Figure 4D and 4E).

Evaluation of Symptoms and Exercise Capacity

NYHA-FC was evaluated in all patients during the follow-up period. Four developed heart failure and underwent LVAD implantation or required intravenous catecholamine support within 1 year after treatment and were considered to be NYHA-FC 4. NYHA-FC score was significantly improved at 1 year after cell-patch implantation in the responder group (class II,

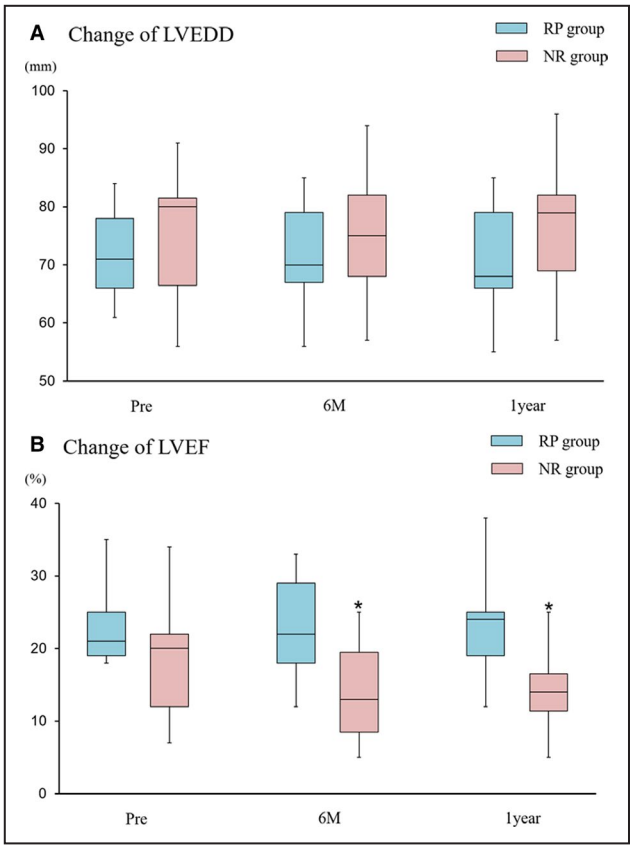

Figure 3. Echocardiographic analysis of cardiac function. **A**, Serial change of left ventricular end-diastolic dimension (LVEDD) in the responder (RP) and nonresponder (NR) groups. **B**, Serial change of left ventricular ejection fraction (LVEF) in the RP and NR groups. Median, interquartile range, and upper and lower extremes are described with box and whisker plots. *Versus pre, P<0.05 (Wilcoxon signed rank test). 6M indicates 6 months.

7 [54%] and class III, 6 [46%] to class II, 12 [92%] and class I, 1 [8%], P<0.05) (Figure 5A), whereas in the nonresponder group, a significant improvement was not seen (class II, 1 [9.1%]; class III, 9 [81.8%]; and class IV, 1 [9.1%] to class II, 5 [45.5%]; class III, 3 [27.3%]; and class IV, 3 [27.3%], P=0.39) (Figure 5B). Exercise capacity was evaluated by a combination of 6MWD test and SAS results. In the responder group, values for both were significantly increased at 1 year after treatment (6MWD, 471 m [370–540 m] to 525 m [425–555 m] [P<0.05]; SAS, 3.5 [2.5–4.5] to 3.5 [2.5–6.5] metabolic equivalents [P<0.05]). Nine of 13 (69.2%) patients in the responder group showed increases in both the 6MWD test and SAS values. In the nonresponder group, the 6MWD test and SAS values were not significantly different after treatment (6MWD, 454 m [367.5–491.5 m] to 457 m [338–521 m] [P=0.81], SAS: 3.5 metabolic equivalents [2.5–3.5 metabolic equivalents] to 2.75 metabolic equivalents [2.5–5.0 metabolic equivalents] [P=1.0]) (Figure 5C and 5D). In the present patients, the SAS, which is determined on the basis of an interview with a physician, had a positive correlation with the quantitatively determined 6MWD test (ρ=0.44, P<0.01). The results of analysis of significance for the 6MWD test and SAS in the responder and nonresponder groups supported these results (responder, P=0.027 and 0.044, respectively; nonresponder, P=0.298 and 0.670, respectively).

BNP Blood Sampling

Serum BNP levels were significantly decreased at 6 months and 1 year after treatment in the nonresponder group (234 pg/mL [76–361 pg/mL] at baseline, 127 pg/mL [60–249 pg/mL] at 6 months, and 116 pg/mL [39–279 pg/mL] at 1 year after treatment [P<0.05]). Furthermore, 10 of 13 (76.9%) of those patients demonstrated a decreased BNP level after undergoing cell-patch implantation. In the nonresponder group, serum BNP was increased at 6 months and 1 year after treatment (541 pg/mL [256–672 pg/mL] at baseline, 726 pg/mL [261–1033 pg/mL] at 6 months, and 598 pg/mL [263–1012 pg/mL] at 1 year after treatment [P<0.05 and 0.12, respectively]) (Figure 6).

Histological Analysis

Preoperatively, a cardiac tissue biopsy was performed in 22 patients to confirm diagnosis. The size of cardiac myocytes in the responder group was lower in that examination as compared with the nonresponder group (17.7±1.1 μm versus 21.1±3.7 μm, P<0.05). Also, interstitial fibrosis was assessed using Sirius red–stained sections, and the percentage of fibrous components in total cardiac tissue was significantly lower in the responder group (21.0±2.0% versus 30.2±2.4%, P<0.05) (Figure 7A and 7B). The expression of 5 histone methylation–related molecules was also immunohistologically assessed in the preoperative cardiac biopsy samples. Histone methylation–related H3K4me3 molecules were found to be expressed at a significantly greater level in the responder group (Figure 7C through 7G).

Univariate and Multivariate Analysis Using a Logistic Regression Model

The result of univariate analysis for response to cell-patch implantation using a logistic regression model is shown in Table 4. We identified 5 significant factors related to a responder of this treatment and chose 3 factors (BNP, PCWP, and H3K4me3) for multivariate logistic regression analysis. BNP was considered as a standard biochemical marker of heart failure, PCWP as a standard marker of heart failure in a pressure study, and H3K4me3 as the most significant variable in histological analysis. A multivariate logistic regression model demonstrated a combination of BNP, PCWP,

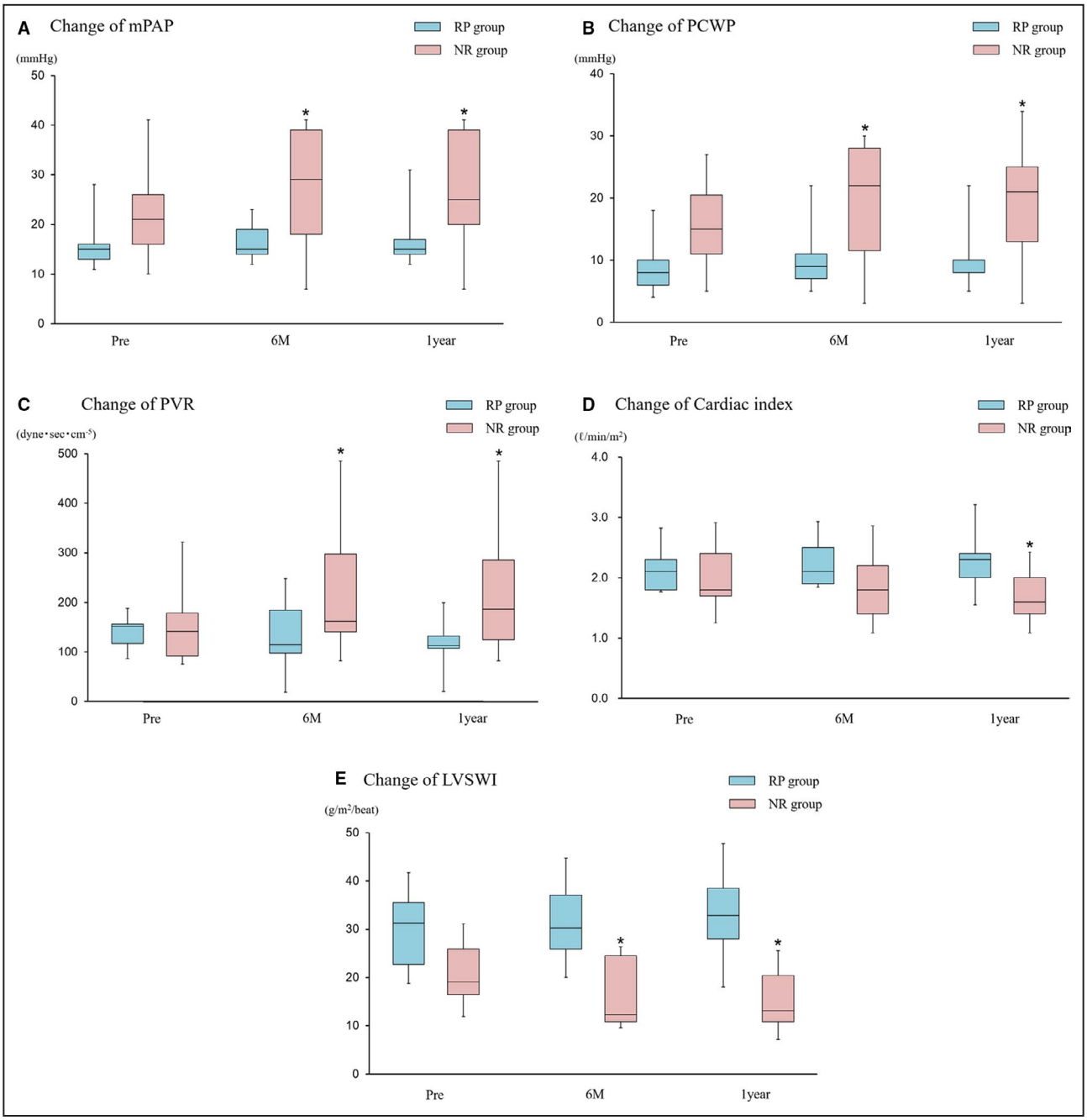

Figure 4. Pressure data evaluated by right heart catheterization.
A, Change of mean pulmonary artery pressure (mPAP) in the responder (RP) and nonresponder (NR) groups. **B**, Change of pulmonary capillary wedge pressure (PCWP) in the RP and NR groups. **C**, Change of pulmonary vascular resistance (PVR) in the RP and NR groups. **D**, Change of cardiac index in the RP and NR groups. **E**, Change of left ventricular stroke work index (LVSWI) in the RP and NR groups. Median, interquartile range, and upper and lower extremes are described with box and whisker plots. *Versus pre, P<0.05 (Wilcoxon signed rank test). 6M indicates 6 months.

and expression of H3K4me3 in a preoperative biopsy sample and strongly predicted a positive response to cell-patch implantation with area under the receiver operating characteristic curve of 0.96 (P<0.0001; BNP/10: odds ratio [OR], 0.96; 95% CI, 0.87–1.07; PCWP: OR, 0.58; 95% CI, 0.28–1.16; H3K4me3: OR, 1.35; 95% CI, 0.98–1.86) (Table 5 and Figure 8).

DISCUSSION

We performed the first clinical trial of autologous skeletal stem cell–patch implantation and evaluated its feasibility, safety, and possible therapeutic efficacy when used as the sole therapy for NIDCM. Fabrication and implantation of a cell patch were successfully

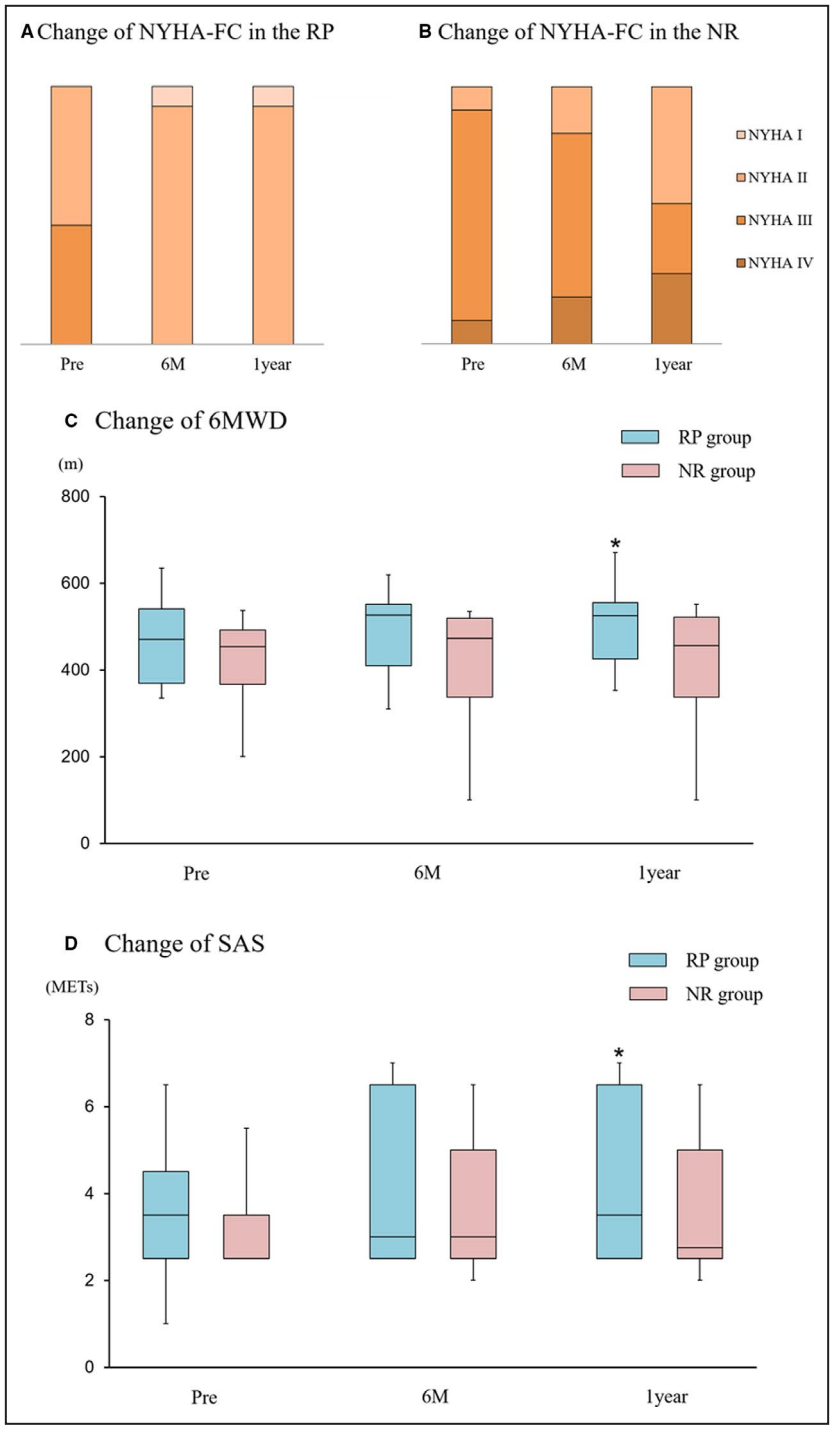

Figure 5. Symptom and exercise capacity.
A and **B**, Changes of symptoms evaluated by New York Heart Association functional class (NYHA-FC) in the responder (RP) and nonresponder (NR) groups. **C**, Changes of exercise capacity evaluated by the 6-minute walk distance (6MWD) test in the RP and NR groups. **D**, Changes of exercise capacity evaluated by the specific activity scale (SAS) in the RP and NR groups. Median, interquartile range, and upper and lower extremes are described with box and whisker plots. *Versus pre, P<0.05 (Wilcoxon signed rank test). 6M indicates 6 months.

performed in all cases, with no procedure-related complications or postoperative lethal arrhythmia in any of the patients.[16]

Although it is common to perform a randomized control study to prove the effectiveness of medications or devices, it may be difficult to elucidate the features

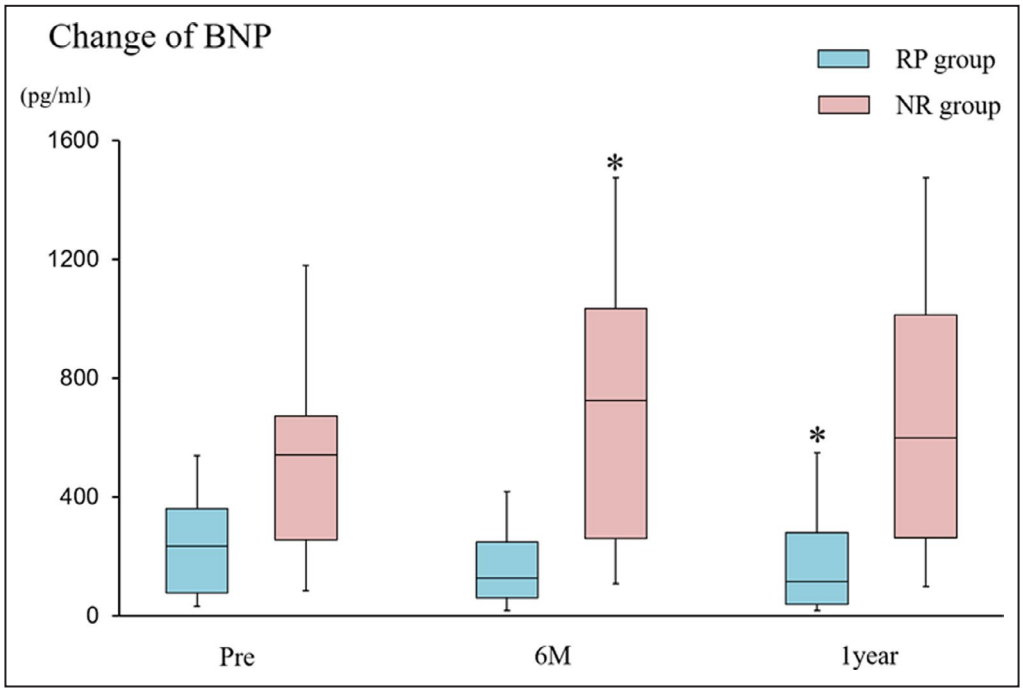

Figure 6. B-type natriuretic peptide (BNP) by blood sampling.
Serial changes in serum level of BNP in the RP and NR groups. Median, interquartile range, and upper and lower extremes are described with box and whisker plots. *Versus pre, P<0.05 (Wilcoxon signed rank test). 6M indicates 6 months.

and true effectiveness of new products developed for intractable and orphan diseases with various causes, and also to select patients using preoperative predictors. For confirming efficacy, it may be crucial to develop new therapeutic armamentaria. Nevertheless, the present results confirmed the feasibility and safety of autologous skeletal stem cell–patch implantation, and therapeutic efficacy was also detected in patients who showed a response to treatment.

Primary End Point for Definition of Responder

When performing a clinical trial of a strategy for treating severe heart failure, it is essential to set an appropriate primary end point for evaluating therapeutic efficacy. In past studies, LVEF was applied for evaluating various therapeutic modalities for heart failure as the primary end point. Rather, therapeutic efficacy of a treatment for patients with heart failure must be evaluated by a comprehensive indicator that includes not only cardiac performance, but also factors such as symptoms, quality of life, and clinical outcomes. In the present study, we set the primary end point as the combination of occurrence of a MACE and incidence of heart failure.

When considering the large medical expense associated with such cases, lowering the incidence of heart failure or hospitalization may be a reasonable end point for evaluation of the efficacy for a specific treatment. In general, NIDCM is characterized as a progressive disease, and patients with Stage C or D heart failure have potential for a MACE and increased incidence of heart failure events.[17] All patients enrolled in the present study were considered to have stable Stage C or D heart failure status under optimal medical therapy. Therefore, we defined the nonresponders to the present cell-patch treatment as patients for whom the treatment failed to prevent progression of disease, with the result being occurrence of a MACE or an increase in the incidence of heart failure. Significant differences of preoperative value related to cardiac condition, especially NYHA-FC, BNP, PCWP, and LVSWI, between the 2 groups suggest our primary end point was also reasonable for evaluation of clinical outcome in patients with heart failure.

Therapeutic Efficacy in Regard to Cardiac Performance, Symptoms, and Exercise Capacity

In the present responder group, cardiac performance, as shown by LVEF representing systolic function and LVEDD, was not changed and found to be maintained at 1 year after treatment. Interestingly, LVSWI and cardiac index evaluated by pressure measurements, considered to be general indicators of cardiac performance, tended to improve after

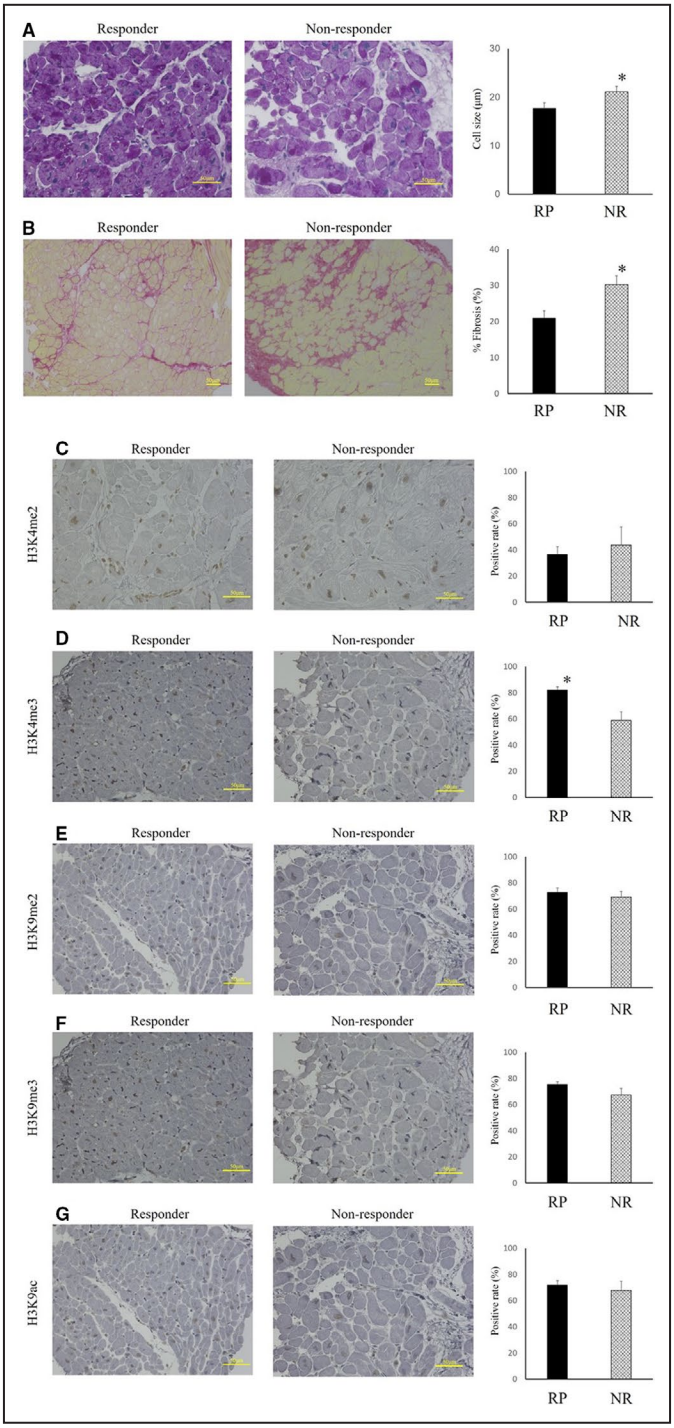

Figure 7. Histological analysis.
A, Myocyte cell size evaluated by periodic acid-Schiff staining. **B**, Interstitial fibrosis evaluated by Sirius red staining. **C** through **G**, Histone modification profiles evaluated by immunohistochemical labeling for each related protein. *Responder (RP) group vs nonresponder (NR) group, $P<0.05$ (Mann-Whitney U test). H3K4me2 indicates histone H3 lysine 4 demethylation; H3K4me3, histone H3 lysine 4 trimethylation; H3K9ac, histone H3 lysine 9 acetylation; H3K9me2, histone H3 lysine 9 demethylation; H3K9me3, histone H3 lysine 9 trimethylation.

Table 4. Univariate Analysis of Predictor of the Cell-Patch Implantation

Variables	Responder	Nonresponder	Univariate Analysis			
			OR	Lower CI	Upper CI	*P* Value
Age, y	53.5±15.4	53.5±9.9	1.00	0.94	1.07	1.00
NYHA II	7 (53.8%)	1 (9.1%)	0.09	0.01	0.88	0.039*
NYHA III	6 (46.2%)	9 (81.8%)				
NYHA IV	0	1 (9.1%)				
Incidence of HF	0.74±0.21	0.64±0.18	1.27	0.37	4.38	0.70
BNP/10	23.3±4.5	56.1±10.0	0.94	0.90	0.99	0.020*
6MWD	468±30	425±33	1.00	1.00	1.01	0.34
Variables by pressure study						
mPAP	15.5±1.2	22.3±2.9	0.86	0.73	1.01	0.064
PCWP	8.5±1.2	15.5±2.1	0.80	0.67	0.97	0.021*
PVR	140±8.6	158±25.6	0.99	0.98	1.01	0.47
LVSWI	30.2±2.1	21.2±2.1	1.19	1.03	1.39	0.019*
Echocardiographic variables						
LVEDD	73.1±2.8	74.9±3.5	0.98	0.91	1.06	0.67
LVEF	23.1±1.7	18.1±2.3	1.12	0.97	1.29	0.11
Histological variables						
Cell size	17.1±1.1	21.1±1.1	0.79	0.61	1.02	0.075
% Fibrosis	19.3±2.8	30.2±2.4	0.85	0.71	1.01	0.072
H3K4me2	36.5±6.0	43.8±13.8	0.99	0.95	1.04	0.70
H3K4me3	82.0±2.5	58.8±6.8	1.18	1.04	1.33	0.0094*
H3K9me2	72.7±3.4	69.1±4.2	1.02	0.95	1.10	0.52
H3K9me3	75.3±2.3	67.5±5.0	1.06	0.97	1.15	0.18
H3K9ac	71.8±3.4	67.8±6.9	1.01	0.96	1.07	0.60

6MWD indicates 6-minute walk distance test; BNP, B-type natriuretic peptide; H3K4me2, histone H3 lysine 4 demethylation; H3K4me3, histone H3 lysine 4 trimethylation; H3K9ac, histone H3 lysine 9 acetylation; H3K9me2, histone H3 lysine 9 demethylation; H3K9me3, histone H3 lysine 9 trimethylation; HF, heart failure; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; LVSWI, left ventricular stroke work index; mPAP, mean pulmonary artery pressure; NYHA, New York Heart Association; OR, odds ratio; PCWP, pulmonary capillary wedge pressure; and PVR, pulmonary vascular resistance.
*P<0.05.

cell-patch treatment. These results suggest that the present treatment enhanced diastolic performance rather than systolic performance.[18] Furthermore, despite the increased cardiac index, indicating an increase in stroke volume, average PCWP was not changed following the procedure. In such cases, the pressure-volume curve during the end-diastolic period demonstrates a downward shift, also suggesting improvement in diastolic performance, especially filling function. On the other hand, in the nonresponder group, we observed increased PCWP and PVR, and decreased LVSWI, leading us to conclude that therapeutic efficacy for cardiac performance was achieved by cell-patch implantation in the responder group. In addition, an improvement in serum BNP level, which is correlated with diastolic function, supported the finding of amelioration of LV diastolic function following skeletal stem cell–patch implantation in the RPs.[19,20] We speculate that this improvement in diastolic performance, especially in regard to filling function and stiffness or reduced PVR, was related to an antifibrotic or angiogenic mechanism enhanced by cell-patch treatment via a cytokine paracrine effect coinciding with preclinical treatment.[21–23] Furthermore, such improvement in global cardiac performance possibly contributed to improved symptoms and exercise tolerance, which were described by the 6MWD test and SAS.

Table 5. Multivariate Analysis of Predictor of the Cell-Patch Implantation

Multivariate Analysis				
Variables	OR	Lower CI	Upper CI	*P* Value
BNP/10	0.96	0.87	1.07	0.49
PCWP	0.58	0.28	1.16	0.12
H3K4me3	1.35	0.98	1.86	0.070

BNP indicates B-type natriuretic peptide; H3K4me3, histone H3 lysine 4 trimethylation; OR, odds ratio; and PCWP, pulmonary capillary wedge pressure.

A limitation of our study is the single-arm design. However, we included all patients receiving optimal medical treatment and in a stable condition at least 4 months before undergoing the cell-patch

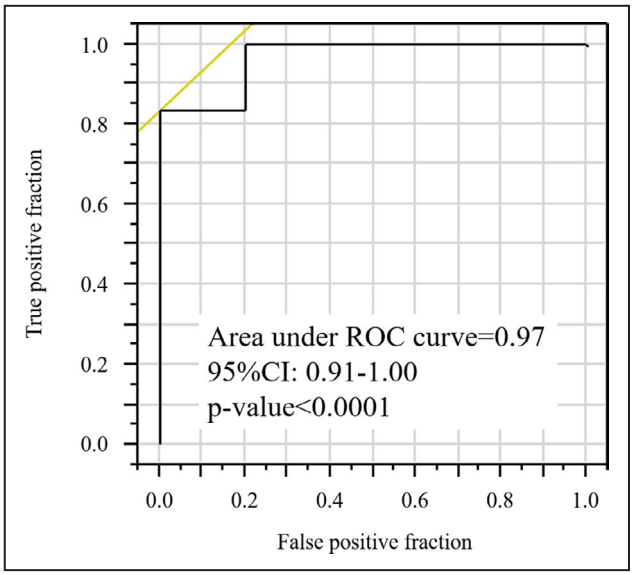

Figure 8. Receiver operating characteristic (ROC) curve analysis.

implantation procedure, and who did not have a change in medication or additional intervention. Therefore, the positive results seen in the responders were considered to be the effects of that sole therapy, whereas the negative results in the nonresponders were because this treatment could not prevent the disease progression or possibly the invasiveness of the procedure. The other limitation of our study was the majority of the patients who were nonresponders did not have a 1-year follow-up in some parameters. However, such nonresponders demonstrated the deterioration of some parameters at 6 months, and they resulted in a MACE. It suggested that these parameters are supposed to be more deteriorated at 1 year if they did not suffer from a MACE. Therefore, this bias should not influence the negative results in the nonresponders. The result of complete-case analysis that agrees with that of the analysis with the last observation carried forward also supported the presumption.

Therapeutic Efficacy in Regard to Freedom From All-Cause Mortality

The long-term survival in the responder group was higher than those in the nonresponder group; however, comparing the survival rate with the control group was consider to be essential for evaluating the impact of this treatment on their prognosis. The SHFM is a well-established and validated model for predicting the prognosis of patients with heart failure, which was calculated by 14 continuous variables and 10 categorical values including clinical, pharmacological, device, and laboratory characteristics.[24] Observed survival rate was superior to predicted survival in the responder group, and it was similar in the nonresponder group. When we assumed predicted survival calculated by the SHFM as that of the untreated control group, these results may suggest that myoblast cell-patch implantation had a good impact on prognosis in selected patients with NIDCM.

Epigenetic Profile Evaluated by Histone Methylation-Related Molecules

In regard to histone profile in distressed myocardium tissue, Kaneda et al reported low levels of expression of H3K4me3 and H3K9me3 in the failing hearts of human subjects. Furthermore, Ito et al noted differences in the expressions of H3K4me3, H3K9me2, and H3K9me3 in cardiac tissues of patients with normal function in pre- and post-LVAD examinations, and also increased expression levels of these histone-related molecules in patients with improved cardiac function from LVAD support.[25,26] Histone methylation is related to genetic expression, and H3K4me3 regulates the activation of transcription and H3K9 the silencing of gene expression, which plays an antagonistic role, whereas H3K4me3 is well expressed in euchromatin with low chromatin aggregation.[27] Kanzaki et al reported a relationship between chromatin aggregation and outcome in patients with heart failure with NIDCM using electron microscopic histological analysis.[28] Histone methylation is considered to be a good epigenetic marker, because it defines not only activated and inactivated chromosome regions, but also predicts clinical outcome. In the present study, we also found a high level of expression of H3K4me3 preoperatively in the responder group, which showed good clinical results. We speculate that residual myocytes possess an ability to produce proteins related to contractile or structural apparatus, or have preserved cell viability to induce functional recovery after treatment in responders. Thus, the level of preoperative expression of histone-related molecules is proposed as a good histological predictor for response to regenerative therapy in patients with NIDCM.

Clinical Predictors for Response to Cell-Patch Implantation

An important aim of our study was to identify factors predictive of response to cell-patch treatment for the purpose of improving clinical outcome by appropriate patient selection. Univariate analysis showed the significance of NYHA-FC, BNP, PCWP, and LVSWI as clinical parameters, and expression of H3K4me3 as a histological parameter. These significant differences in preoperative clinical parameters suggested that more

affected patients were less likely to respond to this cell-patch treatment.

Multivariate analysis revealed that a combination of BNP, PCWP, and H3K4me3 strongly predicted the responders of this treatment. Serum BNP is a widely applicable biomarker, reflecting both the systolic and diastolic function and predicting the long-term outcome of chronic heart failure. PCWP reflects LV end-diastolic pressure, which is correlated with LV wall stress and predicts the progression of LV remodeling.[29] Thus, this parameter may be useful as a predictor, because it is correlated to both LV remodeling and diastolic performance, as noted above.[30] PCWP and H3K4me3 were evaluated by right heart catheterization and cardiac biopsy, which are essential to diagnose the cause and severity of heart failure in patients with NIDCM. Therefore, use of these parameters are reasonable for predicting the responder of cell-patch implantation in the clinical setting.

In our cohort, 2 cases of dHCM were included, and all of them were considered to be nonresponders and resulted in death attributable to LVAD complication. Despite dHCM resembling idiopathic DCM in phenotypic characteristics with ventricular cavity dilation and systolic impairment, patients with dHCM were more symptomatic and demonstrated higher rates of systolic dysfunction and markedly worse prognosis in comparison with patients with idiopathic DCM.[31] Therefore, selection of patients with dHCM with the same criteria of idiopathic DCM may have led to worse clinical outcomes of patients with dHCM in this study. Further study is mandatory for determining which strategy is suitable for eliminating the patients with dHCM or developing different criteria for patients with dHCM in myoblast cell-patch implantation.

There are limited studies introducing cell-based cardiac regenerative therapy for patients with NIDCM. The POSEIDON-DCM (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis in Dilated Cardiomyopathy) study demonstrated the safety of transendocardial injection of human mesenchymal stem cells for treating patients with NIDCM. Comparing the present study to the POSEIDON-DCM study, patients' characteristics were similar, and the allogenic human mesenchymal stem cell group was superior to the autologous human mesenchymal stem cell group about efficacy.[32] However, a limitation of this study was also the lack of a placebo group. Kawamura et al reported the efficacy of human-induced pluripotent stem cell–derived cardiomyocytes (hiPS-CMs) for a porcine ischemic cardiomyopathy model using the cell-patch technique. They suggested that the possible mechanism of hiPS-CMs was not only paracrine effect (mainly angiogenesis), but a direct effect of engrafted hiPS-CMs, which induced cardiomyogenesis in the failing heart. Therefore, prolonging cell survival after implantation might be necessary to obtain long-standing therapeutic effects of the hiPS-CM patch.[33] They also reported a cell delivery system using the combination of an omentum flap and cell patch for enhancement of the survival of hiPS-CMs.[34] In this study, autologous skeletal stem cell–patch implantation did not show the significant efficacy in nonresponders of NIDCM. Further study may be needed to elucidate the optimal cell source and cell delivering system for the patients with severely deteriorated cardiac function.

CONCLUSIONS

The results of our clinical trial demonstrated that skeletal stem cell–patch implantation can provide functional recovery and good clinical outcome in selected patients with NIDCM with preoperatively preserved diastolic function and the ability of protein synthesis in myocytes, as well as safety and feasibility of the procedure.

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